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Anti-Tubulin Labeling Reveals Ampullary Neuron Ciliary Bundles in Opisthobranch Larvae and a New Putative Neural Structure Associated With the Apical Ganglion

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Abstract. This investigation examines tubulin labeling associated with the apical ganglion in a variety of planktotrophic and lecithotrophic opisthobranch larvae. Emphasis is on the ampullary neurons, in which ciliary bundles within the ampulla are strongly labeled. The larvae of all but one species have five ampullary neurons and their associated ciliary bundles. The anaspid *Phyllaplysia taylori,* a species with direct development and an encapsulated veliger stage, has only four ampullary neurons. The cilia-containing ampulla extends to the pretrochal surface *via* a long, narrow canal that opens to the external environment through a very small pore (0.1 μm diameter). Cilia within the canal were never observed to project beyond the opening of the apical pore. The ampullary canals extend toward and are grouped with the ciliary tuft cells and remain in this location as planktotrophic larvae feed and grow. If, as has been reported, the ciliary tuft is motile, the pores may be continually bathed in fresh seawater. Such an arrangement would increase sensitivity to environmental chemical stimuli if the suggested chemosensory function of these neurons is correct. In general, ciliary bundles of newly hatched veligers are smaller in planktotrophic larvae than in lecithotrophic larvae. In planktotrophic larvae of *Melibe leonina,* the ciliary bundles increase in length and width as the veligers feed and grow. This may be related to an increase in sensitivity for whatever sensory function these neurons fulfill. An unexpected tubulin-labeled structure, tentatively called the apical nerve, was also found to be associated with the apical ganglion. This putative nerve extends from the region of the visceral organs to a position either within or adjacent to the apical ganglion. One function of the apical nerve might be to convey the stimulus resulting from metamorphic induction to the visceral organs.

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Abbreviations: AG, apical ganglion; AN, apical nerve; MFSW, Millipore-filtered seawater; SCP, small cardioactive peptide.

Introduction

The planktonic larval stages of marine invertebrates are confronted with the need to metamorphose in a habitat appropriate for successful juvenile and adult life. However, in an ocean covering over 70% of the earth's surface, it is nearly always the case that far less than 1% of the available habitats will meet this need. Thus, the larvae of many species have evolved the ability to "delay" metamorphosis (*e.g.,* Kempf, 1981; Kempf and Hadfield, 1985; Pechenik and Eyster, 1989; Pechenik, 1990; Pechenik and Cerulli, 1991) until they sense a chemical inductive cue (the inducer) that identifies their presence in a suitable habitat (*e.g.,* Crisp, 1974; Kriegstein *et al.,* 1974; Hadfield, 1978; Harrigan and Alkon, 1978; Morse and Morse, 1984; Hadfield *et al.,* 2001; Harder *et al.,* 2002a,b; Zhao and Qian, 2002). Pires *et al.* (2000) point out that many larvae are initially unresponsive to the inducer, so the ability to sense it must develop during the planktonic larval period. The apical ganglion is a larval structure that was previously suggested to serve this purpose (Bonar, 1978; Chia and Koss, 1984) and is now known to do so (Hadfield *et al.,* 2000).

Conklin (1897) provides what is perhaps the first description of the apical ganglion (AG) in an invertebrate larva. Bonar (1978) presents the seminal ultrastructural analysis of the AG in larvae of the aeolid nudibranch *Phestilla sibogae.*

He describes it as being composed of three cell types. Chia and Koss (1984) give a more detailed account of AG ultrastructure in larvae of the nudibranch *Rostanga pulchra* and provide the terminology currently used for the cell types of this organ (*i.e.*, ampullary cells, parampullary cells, and ciliary tuft cells). Both Bonar (1978) and Chia and Koss (1984) describe some of the AG cell types as sensory neurons, with Bonar (1978) suggesting that the ampullary cells (hereinafter ampullary neurons) might be responsible for sensing the inductive cue for metamorphosis. Chia and Koss (1984) concur on this point, but also note that more than one sensory cell type is present, thus the AG probably has more than one sensory function.

Recent investigations using immunohistochemical and neurophysiological techniques allow the neurochemical and possible functional properties of the AG cell types to be determined (*e.g.*, Kempf *et al.*, 1997; Marois and Carew, 1997a,b,c; Leise and Hadfield, 2000; Dickinson and Croll, 2003). Kempf *et al.* (1997) and Marois and Carew (1997b, c) analyze the serotonergic and other components of the AG's neuronal circuitry in detail. Their results indicate that the sensory serotonergic neurons of the AG are involved in modulating muscular and ciliary activity in the velum. Kempf *et al.* (1997) also suggest that these AG components may form a compensatory system of control that would allow the larva to orient each velar lobe relative to changes in the other.

Experiments by Leise and Hadfield (2000) demonstrate that in larval *P. sibogae* the central nervous system responds to the inductive cue with a change in the activity pattern of action potentials. Further investigations by Hadfield *et al.* (2000) provide crucial insight into at least one sensory function of the AG. Using photoablation of AG cells or cell parts that are stained by the vital dye DASPEI, they demonstrate that the AG contains the primary receptor for the chemical cue that induces metamorphosis in nudibranch (and possibly other types of spiralian) larvae. Hadfield *et al.* (2000) suggest that DASPEI may be staining the ampullary neurons described in earlier research (*e.g.*, Bonar, 1978; Chia and Koss, 1984) and that these cells may be the primary receptor for the inductive cue; as yet, however, conclusive evidence is lacking.

All previous examinations of the AG ampullary neurons have been ultrastructural. While this work provides information on the cytology of these neurons and some insight into their structural relationships with other cells of the AG (Bonar, 1978; Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997a), ultrastructural examinations are time-consuming, difficult, and subject to potentially substantial artifacts from shrinkage and other problems associated with dehydration and embedding. A means of examining ampullary neurons in whole larvae, using methods that eliminate such difficulties, would be useful. The ampullary neurons in a variety of invertebrate larvae have characteristics that suggest a combination of confocal imaging and fluorescently tagged tubulin antibodies might be useful for identifying them in whole-mount preparations and examining their morphology and associated structures for clues about function. First, ampullary neurons are characterized by thick, dense bundles of internalized, modified cilia (Bonar, 1978; Chia and Koss, 1984; Lacalli, 1981, 1982; Uthe, 1995; Kempf *et al.*, 1997; Marois and Carew, 1997a; Schaefer and Ruthensteiner, 2001; Page, 2002; Ruthensteiner and Schaefer, 2002). Second, they are the only cells in the AG that contain internalized dense, ciliary bundles (Bonar, 1978; Chia and Koss, 1984; Kempf *et al.*, 1997; Page and Parries, 2000). Third, they are adjacent to easily identified, serotonergic parampullary neurons (Kempf *et al.*, 1997; Marois and Carew, 1997a, b). This paper describes the successful use of a tubulin antibody to identify the ampullary neurons in opisthobranch larvae as well as a new structure associated with the AG. Whereas previous studies have dealt mainly with newly hatched larvae, we have also examined later stages in the development of planktotrophic larvae.

Materials and Methods

Adults of the nudibranch *Berghia verrucicornis* (Aeolidiidae) were collected in the Florida Keys and maintained in laboratory culture at Auburn University, Alabama, using the methods of Carroll and Kempf (1990). Those of the nudibranchs *Melibe leonina*, *Tritonia diomedea* (Tethyidae), *Armina californica* (Arminidae), *Janolus fuscus* (Zephyrinidae), and *Phyllaplysia taylori* (Aplysiidae) were collected in the waters of the San Juan Islands and maintained in flow-through seawater tables at the University of Washington's Friday Harbor Laboratories. Egg masses of the nudibranch *Phestilla sibogae* (Aeolidiidae) were kindly provided by Dr. M. G. Hadfield, Kewalo Marine Laboratory, Pacific Biomedical Research Center, University of Hawaii. Of these, the embryo of *P. taylori* undergoes direct development with an encapsulated, embryonic veliger stage, and the newly hatched larvae of *B. verrucicornis* and *P. sibogae* are lecithotrophic and ready to metamorphose a few days after hatching (Bonar and Hadfield, 1974; Harris, 1975; Carroll and Kempf, 1990). The other species all have planktotrophic larvae that must undergo a period of feeding and growth in the plankton before they reach metamorphic competence. Larvae examined were either decapsulated veliger stages (*P. taylori*), newly hatched (*B. verrucicornis*, *P. sibogae*, *J. fuscus*, *A. californica*), or veligers that were either newly hatched or at subsequent stages of growth during planktonic feeding in culture (*M. leonina*, *T. diomedea*).

Egg masses of all species were cultured in glass or plastic beakers with either artifical seawater (Reef Crystals, Aquarium Systems) at about 30 ppt from well-established aquaria (*B. verrucicornis*, *P. sibogae*) or fresh seawater (all other species and *P. sibogae*). In all cases, 0.45-μm Millipore-

filtered seawater (MFSW) was used and cultures were aerated (Kempf *et al.*, 1997; Miller and Hadfield, 1986). Planktotrophic larvae of *M. leonina* and *T. diomedea* were cultured in the laboratory using previously published methods (Kempf and Willows, 1977; Bickell and Kempf, 1983).

In preparation for fixation, veliger-stage embryos of *P. taylori* were decapsulated by tearing apart egg masses with forceps and collecting larvae that were released from their capsules. Larvae of the other species were collected either at hatching or at various stages of growth during feeding in culture. All embryos or larvae were decalcified, as described by Pennington and Hadfield (1989), in an MES buffered, ~pH 5.5, MBL seawater solution for a few hours to overnight in a refrigerator or a 17 °C incubator. Decalcified veliger stages of *P. taylori* were transferred directly to fixative (see below). Larval stages of the other species were relaxed in a 1:3 mixture of chlorotone-saturated seawater and MFSW at room temperature (Bonar and Hadfield, 1974). Embryos and larvae were fixed in 4% paraformaldehyde containing 0.2 *M* Millonig's phosphate buffer and 0.14 *M* NaCl, rinsed in 20 m*M* phosphate-buffered saline (PBS), permeabilized by ethanol dehydration and rehydration, and stored in a refrigerator, all as described for light microscopy in Kempf *et al.* (1997).

Antibody labeling was performed as described by Kempf *et al.* (1997). Primary antibodies were mouse anti-acetylated tubulin (Sigma, Cat. # 6-11B-1) diluted 1:500, rabbit anti-serotonin (Diasorin) diluted 1:1000, and as a positive control, mouse anti-small cardioactive peptides (anti-SCP, Masinovsky *et al.*, 1988) diluted 1:20. Secondary antibodies were either goat anti-mouse or anti-rabbit IgG (MT Biomedicals) conjugated to fluorescene (FITC) or rhodamine (RITC), or donkey anti-mouse or anti-rabbit IgG (Molecular Probes) conjugated to Alexafluor 488 or 594. Tissues labeled with one or more primary antibodies were incubated in the appropriate secondary antibody or antibodies at dilutions of 1:1000 for goat anti-mouse or anti-rabbit IgG and 1:200–1:400 for donkey anti-mouse or anti-rabbit IgG. Antibodies were diluted in 20 m*M* PBS containing 5% heat-inactivated goat serum, 0.1% NaN_3, and 0.1% Triton X-100. Larvae unlabeled with primary antibody were incubated in secondary antibody or antibodies only as a negative control. In addition to the species already mentioned, we also attempted to label the ampullary cilia in larvae of the bivalve *Crassostrea virginica* and the prosobranchs *Ilyanassa obsoleta* (kindly provided by Dr. E. Leise), *Trichotropis cancellata*, *Crepipatella dorsalis*, and *Fusitriton oregonensis* with the Sigma anti-acetylated tubulin antibody.

Labeled larvae were mounted under coverslips in either DPX mountant (Electron Microscopy Sciences) after ethanol dehydration or in a glycerol mounting medium containing 5% *n*-propyl gallate (Giloh and Sadat, 1982). Larvae of *B. verrucicornis* and *P. sibogae* were examined with a Bio-Rad MRC-1024 confocal, laser-scanning system mounted on a Zeiss Axioskop microscope. Larvae of *M. leonina*, *T. diomedea*, *J. fuscus*, *A. californica*, and *P. taylori* were examined on a Bio-Rad Radiance confocal, laser scanning system mounted on a Nikon Eclipse E800 microscope. Both 40× dry and 40× and 60× oil immersion objectives were used to collect data with Bio-Rad Lasersharp 2000 software (ver. 5.2). Images were processed for contrast and color with Adobe Photoshop.

Newly hatched larvae of *T. diomedea* were relaxed and then fixed in phosphate-buffered 2.5% glutaraldehyde with post-fixation in 2% bicarbonate-buffered osmium tetroxide for ultrastructural examination. Details of the procedures and methods for fixation, embedment, sectioning, and staining are described by Page (1995). In the region of the apical ganglion where the ampullary neurons are located, serial sections were cut to locate the pores of the ampullary canals. Stained sections were examined and images collected using a Hitachi 7000 electron microscope. Images of the ampullary pores were processed for contrast using Adobe Photoshop.

To assess differences in the size of the ciliary bundles of ampullary neurons in newly hatched larvae of various species and in 13-day post-hatch larvae of *M. leonina*, the length and width of the central and lateral ciliary bundles were measured using maximum intensity projections of the original data from the serial optical sections and the line function in Bio-Rad Lasersharp analysis software. Only larvae presenting a near-frontal view of the ciliary bundles were measured. Widths were measured across the center of each ciliary bundle. Lengths were measured from the base of the ampulla to the point at the apex where the bundle narrows into a canal that extends to the pretrochal surface (Fig. 1). If the ciliary bundles were bent, the length was measured as the sum of the connected lengths of the straight portions of the bundle (Fig. 1). The significance of differences in length and width was tested using a two-way analysis of variance run on the SAS statistical software package (ver. 8.2).

Results

Ampullary neurons

The apical ganglion (AG) of the larvae of all opisthobranch species and developmental stages examined had ampullary neurons, the ciliary bundles of which were labeled by Sigma's anti-acetylated tubulin antibody. In each species, the ciliary bundles could be seen centrally positioned between the velar lobes and beneath the pretrochal epidermis (Fig. 2A). In all species except *Phyllaplysia taylori*, five labeled ciliary bundles were clearly evident at hatching and in subsequent larval stages. These ciliary bundles were grouped as two lateral pairs to the left and right of a central ciliary bundle (Fig. 2A–E, G–I). In *P. taylori*, the central ciliary bundle appeared to be missing, but the four ciliary bundles of the lateral pairs were present (Fig. 2F). In

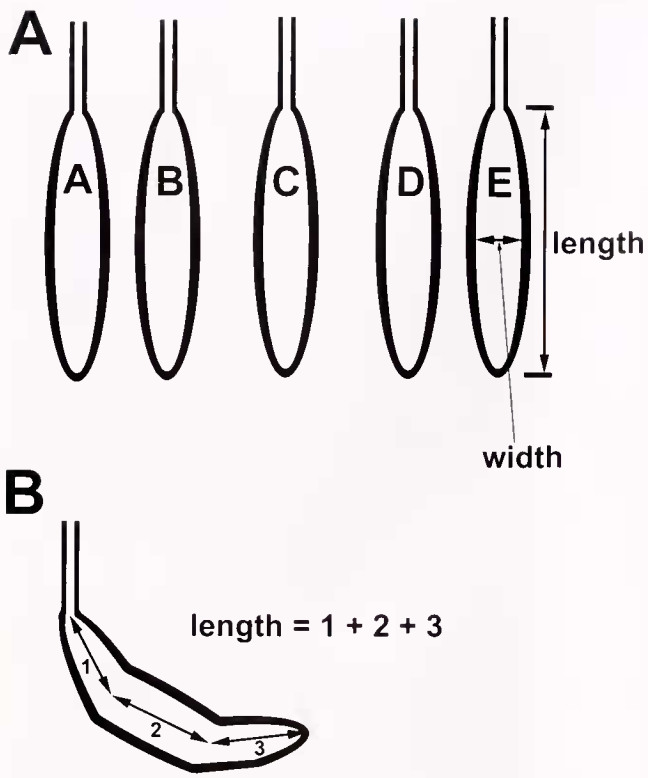

Figure 1. Measurement of ampullary neuron ciliary bundles. (A) Width and length in unbent ciliary bundles were measured as shown. (B) Length in bent ciliary bundles was measured as the sum of the linear components of the bent bundle.

newly hatched larvae of planktotrophic species, it was often difficult to discern the projection of labeled bundle cilia into the ampullary canal that extends to the pretrochal surface (Fig. 2D, G, H); however, this projection was clearly evident, as revealed by intense tubulin labeling, in newly hatched larvae of lecithotrophic species (Fig. 2B, C) and in later stage planktotrophic larvae of *Melibe leonina* (*e.g.*, Fig. 2E). Later planktotrophic larval stages of *Armina californica* and *Janolus fuscus* were not examined. Sigma's anti-acetylated tubulin antibody did not label ampullary cilia in larvae of the bivalve *Crassostrea virginica* or the prosobranchs *Ilyanassa obsoleta, Trichotropis cancellata, Crepipatella dorsalis,* and *Fusitriton oregonensis,* though other cilia, such as those of the velum and digestive tract, were labeled.

The projection of ampullary cilia within the ampullary canal extending to the pretrochal surface was clearly evident in newly hatched lecithotrophic larvae of *Berghia verrucicornis* (Fig. 2B) and *Phestilla sibogae* (Fig. 2C), as well as in later stage planktotrophic larvae of *M. leonina* (Fig. 2E). Both at hatching and in the later stage planktotrophic larvae, the ampullary canals of all the ampullary neurons were closely grouped around the central ciliary tuft cells (*e.g.*, Fig. 2B, C, E). Despite a previous report (Bonar, 1978), it was not possible to detect cilia projecting beyond the opening of the ampullary pore. Transmission electron microscopic examination of ampullary neurons in newly hatched larvae of *Tritonia diomedea* clearly revealed the ampullary pore (Fig. 3) and indicated that the ampullary cilia do not extend above the pretrochal surface at hatching.

Sufficient tubulin antigen was sometimes present in the perikarya of the ampullary neurons to make them visible in confocal optical sections. The perikarya were always closely grouped and had basal nuclei (Fig. 4A, B). The ciliary bundles within each ampullary neuron were in a cylindrical pocket that extended toward the pretrochal surface. The final portion of the ampulla consisted of a long, thin, tubular conduit (the ampullary canal) that extended between the ciliary bundle and the apical pore at the pretrochal surface (Fig. 2B, C, E). Measurement of appropriately oriented neuronal perikarya in Figure 4A and B gave maximum cell widths of 7.0 ± 1.3 μm (n = 3) for 13-day-old *M. leonina* and 6.6 ± 0.7 μm (n = 3) for 15-day-old *T. diomedea.* Total cell lengths (perikaryon + ciliary bundle) were 21.2 ± 1.4 μm (n = 2) for *M. leonina* and 18.1 ± 0.8 μm (n = 3) for 15-day-old *T. diomedea.*

Measurement of ciliary bundles (Table 1) in ampullary neurons of newly hatched larvae revealed that their length ranged from 8.5 ± 1.1 μm (*T. diomedea*) to 12.7 ± 1.5 μm (*P. sibogae*) and width from 1.2 ± 0.2 μm (*T. diomedea, A. californica*) to 2.6 ± 0.6 μm (*P. sibogae*). The planktotrophic larvae of *T. diomedea* had the shortest ciliary bundles, while those of the lecithotrophic larvae of *P. sibogae* were the longest.

When ciliary bundle lengths of the five ampullary neurons in larvae of *M. leonina* were compared within groups of newly hatched (F = 0.06; P = 0.9938) or 13-day-old (F = 1.03; P = 0.4000) larvae, there were no significant differences. The only significant difference in a comparison of ciliary bundle widths for the five ampullary neurons in newly hatched larvae was between the central bundle and lateral bundles (F = 8.55; $P < 0.0001$). There were no significant differences in length or width among ciliary bundles of 13-day-old larvae (F = 0.30; P = 0.8755). A comparison of both ciliary bundle length and width between newly hatched and 13-day-old larvae of *M. leonina* revealed that bundles were significantly longer (F = 93.97; $P < 0.0001$) and wider (F = 264.37; $P < 0.0001$) in 13-day-old larvae.

Positive controls using anti-SCP had typical labeling (Kempf *et al.*, 1987) of SCP-containing neurons and axons in central nervous system ganglia, commissures, and connectives and no labeling in the region of the apical ganglion (Fig. 4C). Larvae incubated in secondary antibody only showed no specific labeling (*e.g.*, Fig. 4D, E). Positive controls using anti-serotonin antibody labeled the characteristic serotonergic neurons and associated axons of the AG, but not the ampullary neurons (Fig. 5A).

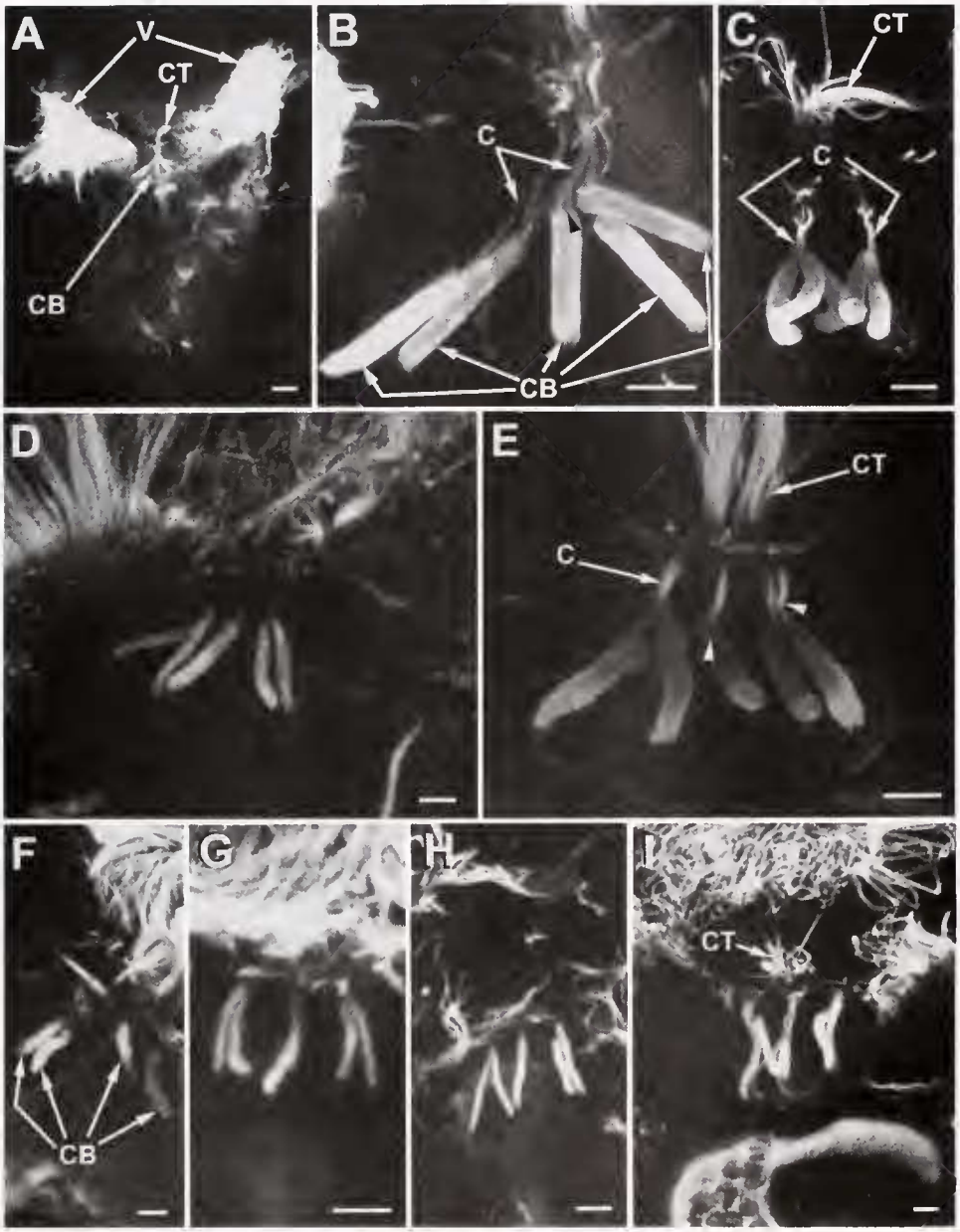

Figure 2. Ciliary bundles of ampullary neurons in seven species of opisthobranchs. Abbreviations: C, labeled cilia; CB, ciliary bundles; CT, ciliary tuft; V, velar lobes. (A) Low-magnification image of a newly hatched, competent larva of *Berghia verrucicornis* in frontal view. The ciliary bundles of the five ampullary neurons lie beneath the ciliary tuft and between the velar lobes. (B) High-magnification image of the five ampullary neurons in a newly hatched, competent larva of *B. verrucicornis*. Note the very long ampullary canals that are defined by the labeled cilia within. In some cases (*e.g.*, arrowhead) anti-tubulin labeling of the cilia suggests that more than one canal may arise from a single ampullary neuron. (C) The ciliary bundles of the five ampullary neurons in a newly hatched, near-competent larva of *Phestilla sibogae*. Note that the canals of the ampullary neurons appear to form two tight groups on the left and right sides of the ciliary tuft. (D) The five ciliary bundles of the ampullary neurons in a newly hatched planktotrophic larva of *Melibe leonina*. As indicated by very weak labeling, few cilia are present in the apical canals of this planktotrophic species at hatching. (E) High-magnification image of the five ciliary bundles in a 13-day-old larva of *M. leonina* (~19 days before competence; Bickell and Kempf, 1983). This larva is a little over halfway to metamorphic competence. As in *B. verrucicornis*, very long apical canals are revealed by labeling of cilia within. Also, as in *B. verrucicornis*, in some instances anti-tubulin labeling of the cilia suggests that more than one canal extends from a single ampullary neuron (arrowheads). (F) The ciliary bundles in a veliger-stage embryo of *Phyllaplysia taylori*. Only four ampullary neurons are present in encapsulated veligers of this species. (G, H) Ciliary bundles of the five ampullary neurons in a newly hatched, planktotrophic larva of *Armina californica* (G) and *Janolus fuscus* (H). (I) Ciliary bundles of the five ampullary neurons in a 15-day-old planktotrophic larva of *Tritonia diomedea*. Scale bars: A = 20 μm; B–I = 5 μm.

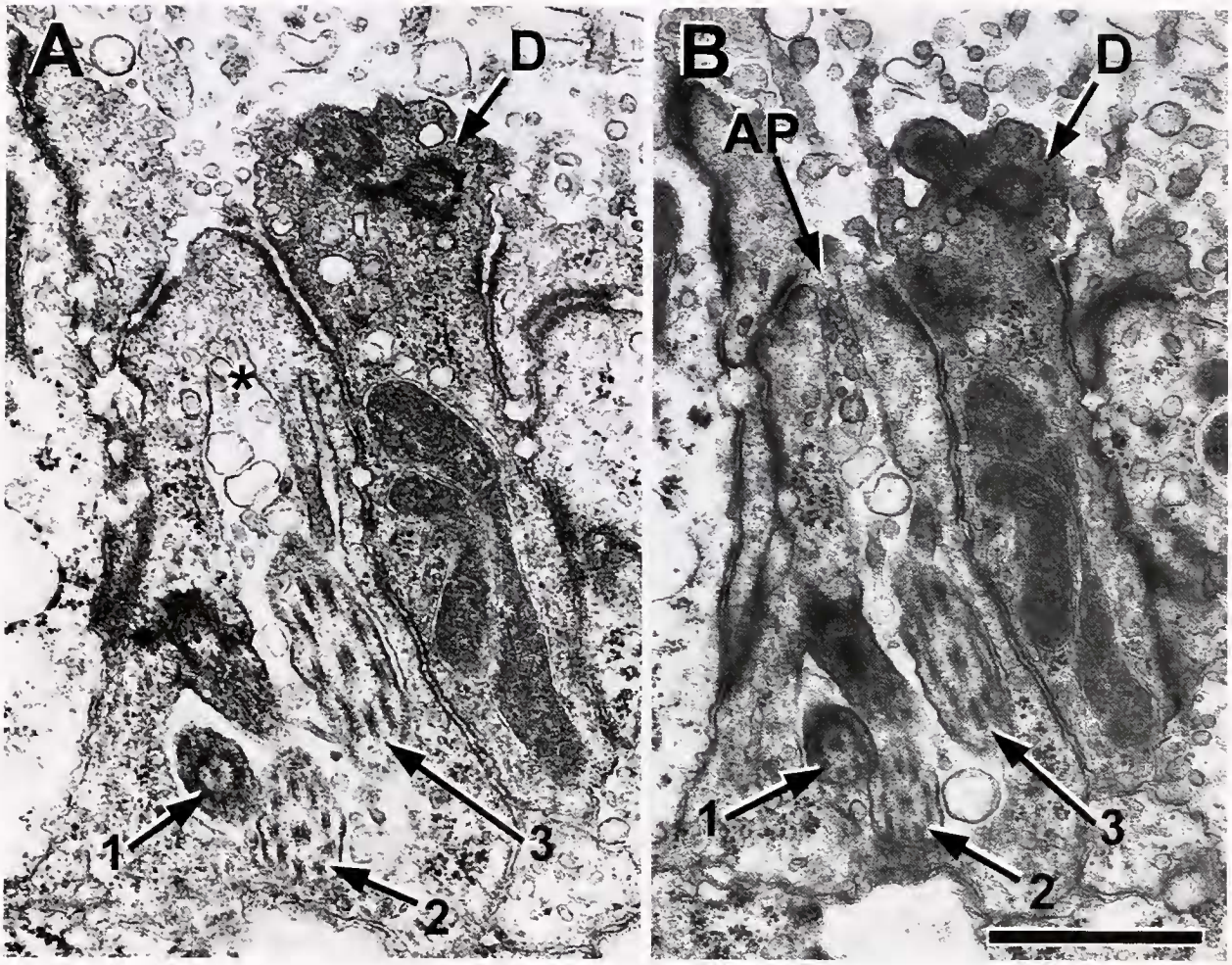

Figure 3. Electron micrographs from the apical ganglion of a newly hatched larva of *Tritonia diomedea*, showing the opening of the ampullary canal at the pretrochal surface that forms the ampullary pore. (A) The asterisk marks the ampullary canal in a section just before its opening at the pretrochal surface. Three cilia (1, 2, 3) within the canal are indicated. The dendrite (D) of a type I parampullary neuron lies directly adjacent to the ampullary neuron. The basal bodies of the two cilia characteristic of this type of dendrite can be seen at its tip. (B) The next section following that in (A) shows the ampullary pore (AP) opening at the pretrochal surface. As is evident, the pore is exceedingly small. Scale bar = 1 μm.

Apical nerve

In addition to the ciliary bundles of ampullary neurons, the Sigma anti-acetylated tubulin antibody also labeled a new structure, tentatively called the apical nerve (AN). This structure was observed in newly hatched planktotrophic larvae of *M. leonina* and newly hatched lecithotrophic larvae of *B. verrucicornis*, as well as in later stages of both *M. leonina* and *T. diomedea*. Larvae of *A. californica*, *J. fuscus*, *P. sibogae*, and *Phyllaplysia taylori* were not specifically examined for the presence of this structure. The best examples of the AN were observed in 13-day-old larvae of *M. leonina*. Serial confocal, optical sections of larvae double-labeled with anti-serotonin and anti-acetylated tubulin revealed that the termini of the AN were dorsal to the serotonergic parampullary neurons and ampullary neurons and either within or associated with the apical ganglion (see Fig. 5A–G). The AN consisted of a long twisted process that originated in the region of the larval viscera and extended to just below the pretrochal epidermis. At its end, beneath the pretrochal epidermis, the process split into three branches that ended in swollen termini (Fig. 5F, G). One branch had a long thin appendage extending from its swollen terminus (Fig. 5 F, G, asterisk-arrow). In 13-day-old larvae of *M. leonina*, the AN could be seen to end dorsal to the ciliary bundles of the ampullary neurons that were closely grouped around the ciliary tuft cells. The serotonergic parampullary neurons were ventral to this grouping, with the dendrites of the lateral, sensory, parampullary neurons extending to the

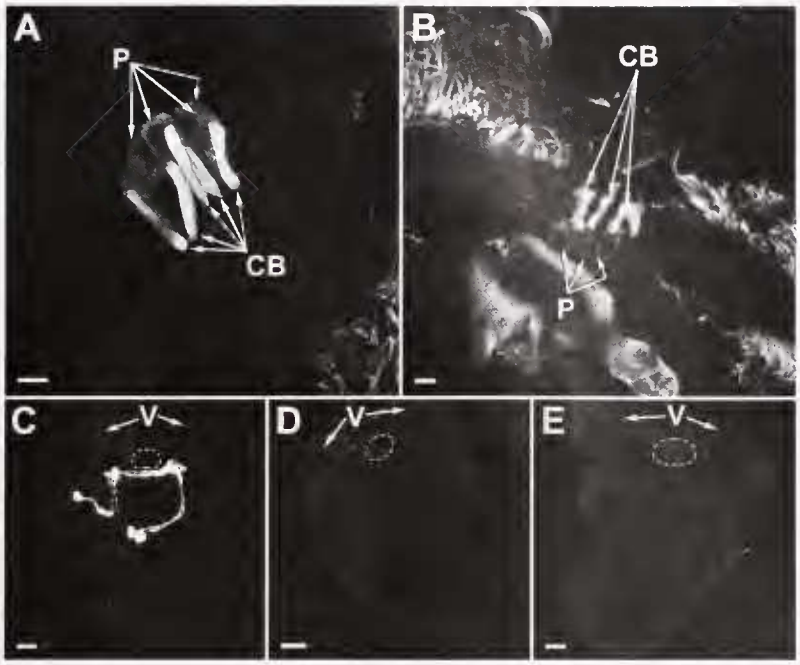

Figure 4. Ampullary neuron perikarya and control labeling. (A) Ampullary neuron perikarya (P) and ciliary bundles (CB) in a 13-day-old larva of *Melibe leonina* (~19 days before competence; Bickel and Kempf, 1983). (B) Ampullary neuron perikarya and ciliary bundles in a 15-day-old larva of *Tritonia diomedea* (~19 days before competence; Kempf and Willows, 1977) (C) Positive control, newly hatched larva of *M. leonina* labeled with a mouse monoclonal antibody that binds to small cardioactive peptides (anti-SCP). Axons crossing the cerebral commissure and extending from posterior neurons on either side of the larva are labeled. The dashed oval indicates the position of the apical ganglion and its associated ampullary neurons. (D) Example of secondary antibody control. Note lack of labeling by secondary, RITC-conjugated, goat anti-mouse IgG antibody in a newly hatched larva of *T. diomedea*. The dashed oval indicates the position of the apical ganglion and its associated ampullary neurons. (E) Example of secondary antibody control. Note lack of labeling by secondary, Alexafluor 594-conjugated donkey anti-mouse IgG antibody in a newly hatched larva of *Armina californica*. The dashed oval indicates the position of the apical ganglion and its associated ampullary neurons. Scale bars: A, B = 5 μm; C, D, E = 10 μm.

pretrochal surface some distance to the left and right of the central ampullary neuron-ciliary tuft cell grouping (Fig. 5A–C, D, E). The general position of the apical nerve relative to the ampullary and serotonergic parampullary type I neurons is shown in Figure 6.

Discussion

Apical ganglion and ampullary neurons

The apical ganglion (AG) of molluscs (Leise, 1996; Lin and Leise, 1996a, b; Marois and Carew, 1997a, b) is also known by a variety of other names, *e.g.,* apical sense organ (Conklin, 1897), apical organ (Pelseener, 1911; Werner, 1955; Tardy, 1974; Page, 1992), cephalic sensory organ (Bonar, 1978; Uthe, 1995), apical sensory organ (Chia and Koss, 1984; Kempf *et al.,* 1997), and apical complex (Tardy and Dongard, 1993). Previous studies on the morphology of this ganglion (Bonar, 1978; Chia and Koss; 1984; Tardy and Dongard, 1993; Uthe, 1995; Kempf *et al.,* 1997; Marois and Carew, 1997a, b, c) suggest that the ampullary and parampullary neurons play a sensory and modulatory role that affects larval behavior. Morphological studies by Kempf *et al.* (1997) and Marois and Carew (1997b, c) indicate that serotonergic sensory neurons in and innervation arising from the apical ganglion may modulate muscle contractility, ciliary activity, or both in the velum of larval opisthobranchs. Recent experiments by Hadfield *et al.* (2000) demonstrate that DASPEI-labeled cellular structures of the apical ganglion are critical for primary recognition of the chemical inductive cue that initiates metamorphosis in larvae of the aeolid mollusc *Phestilla sibogae*. These structures have not yet been identified, but Hadfield *et al.* (2000) suggest that they may be the ampullary neurons, which are located in the apical ganglion and are morphologically similar to putative chemoreceptors in the cephalopod olfactory organ (Emory, 1975, 1976; Bonar, 1978). If this is correct, the organization of the pores of the ampullary neurons around the bases of the ciliary tuft cells (as revealed by our anti-tubulin labeling) may be important. This juxta-

Table 1

Length and width of ciliary bundles in ampullary neurons

Species* P, Planktotrophic L, Lecithotrophic E, Embryonic	Ciliary bundle length† Newly hatched	Ciliary bundle length† 13 d post-hatch	Ciliary bundle width† Newly hatched	Ciliary bundle width† 13 d post-hatch
Melibe leonina	11.6 ± 1.0	14.1 ± 1.4	1.8 ± 0.2	2.5 ± 0.3
P	(9, 45)	(9, 45)	(11, 54)	(11, 54)
Tritonia diomedea	8.5 ± 1.1	—	1.2 ± 0.2	—
P	(8, 28)		(8, 29)	
Janolus fuscus	9.5 ± 1.3	—	1.4 ± 0.2	—
P	(4, 16)		(4, 15)	
Armina californica	8.8 ± 0.9	—	1.2 ± 0.2	—
P	(4, 16)		(4, 18)	
Berghia verricicornis	11.1 ± 1.0	—	2.1 ± 0.3	—
L	(4, 10)		(3, 9)	
Phestilla sibogae	12.7 ± 1.5	—	2.6 ± 0.6	—
L	(3, 5)		(2, 4)	
Phyllaplysia taylori	10.3 ± 0.3	—	2.2 ± 0.2	—
L, E	(1, 4)		(1, 4)	

* All stages labeled were hatched larval stages except for those of *Phyllaplysia taylori*, which were veliger stage embryos. *P. taylori* is a direct-developing species that has an encapsulated embryonic veliger but lacks a free-living larval stage. This species hatches as a fully metamorphosed slug.

† Values (in micrometers) are mean ± standard deviation. The first number in parentheses is the number of larvae in which measurements were made. The second number is the total number of individual ciliary bundles that were measured.

position with the ciliary tuft cells is maintained throughout larval development. Since the ciliary tuft appears to be motile (Page and Parries, 2000; unpubl. obs.), it may act to constantly bathe the ampullary pores in fresh seawater, thus enhancing their exposure to dissolved metamorphic inducer when a larva encounters a suitable habitat for the juvenile and adult.

Using figure 2 in Hadfield *et al.* (2000), we determined that the DASPEI-labeled structures that they describe are about 2 μm in diameter. Our results provide a width of about 7 μm and a length of 18–21 μm for ampullary neurons in 13-day-old larvae of *Melibe leonina* and 15-day-old larvae of *Tritonia diomedea*. Since our measurements of ciliary bundles in these species and in *P. sibogae* are similar, we would expect the same for the size of ampullary neurons. This being the case, if the DASPEI labeling reported by Hadfield *et al.* (2000) is associated with the ampullary neurons, then only a small portion of each of these cells, presumably a cytoplasmic "cloud" of mitochondria, was labeled. Nevertheless, photoablation of DASPEI-labeled structures in the AG (Hadfield *et al.*, 2000) is still sufficient to eliminate inductive sensing ability and demonstrate that the AG contains the primary receptor or receptors for the inductive cue.

Ciliary bundle length and width varied among newly hatched larvae of different species. Those with lecithotrophic development (including encapsulated veligers of direct-developing species) usually had the longest and widest ciliary bundles—that is, *P. sibogae, Berghia verrucicornis, Phyllaplysia taylori;* however, this was not an absolute rule since the ciliary bundles in newly hatched *M. leonina* were similar in length to or longer than those in species with lecithotrophic development. As larvae of *M. leonina* grew, their ciliary bundles increased in length and width. In larvae cultured for 13 days, ciliary bundles were longer than at hatching and had increased in width such that they were similar to those of newly hatched lecithotrophic larvae. That being the case, the differences in ciliary bundle length and width observed at hatching between lecithotrophic and planktotrophic larval stages appear to be related to the fact that planktotrophic larvae are not as well developed at hatching (see Table 2) rather than to intrinsic and perhaps functional differences in the ampullary neurons of different species. The increase in length and width of the ciliary bundles as larvae of *M. leonina* grow toward metamorphic competence suggests either that the cilia increase in length and width during larval growth or that new, and possibly longer, cilia are added to the bundle. If the ampullary neurons are the primary receptor for the metamorphic inducer, this increase in length and width of the bundles may reflect an increase in sensitivity in preparation for the metamorphic event.

Previous investigations (Bonar, 1978; Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000) describe the ampullary neurons as having a tapering ampulla that extends to the pretrochal surface and, in some cases, illustrate it as such (Bonar, 1978; Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000). Only Chia and Koss (1984) describe the ampulla's connection with the pretrochal surface as a "long narrow channel or neck" similar to what we observed in later stage planktotrophic larvae and newly hatched lecithotrophic larvae. We suspect this discrepancy in structure between different species is an artifact caused by shrinkage during fixation and embedding for ultrastructural examination. In addition, it is likely that the ampulla's canal is shorter in newly hatched planktotrophic larvae than in later stage larvae of the same species.

The ampullary pore is very small, on the order of 0.1 μm in diameter (see Fig. 3). It can be visualized only in well-fixed tissue that has good preservation of membranes and an absence of excessive shrinkage. Bonar (1978) reports that cilia of the ampullary neurons in a nudibranch veliger extend through the canal (neck) of the ampulla and also through and above the pore at the pretrochal surface. At the opposite extreme, Schaefer and Ruthensteiner (2001) and Ruthensteiner and Schaefer (2002) found no evidence of an external pore for ampullary neurons in a selection of

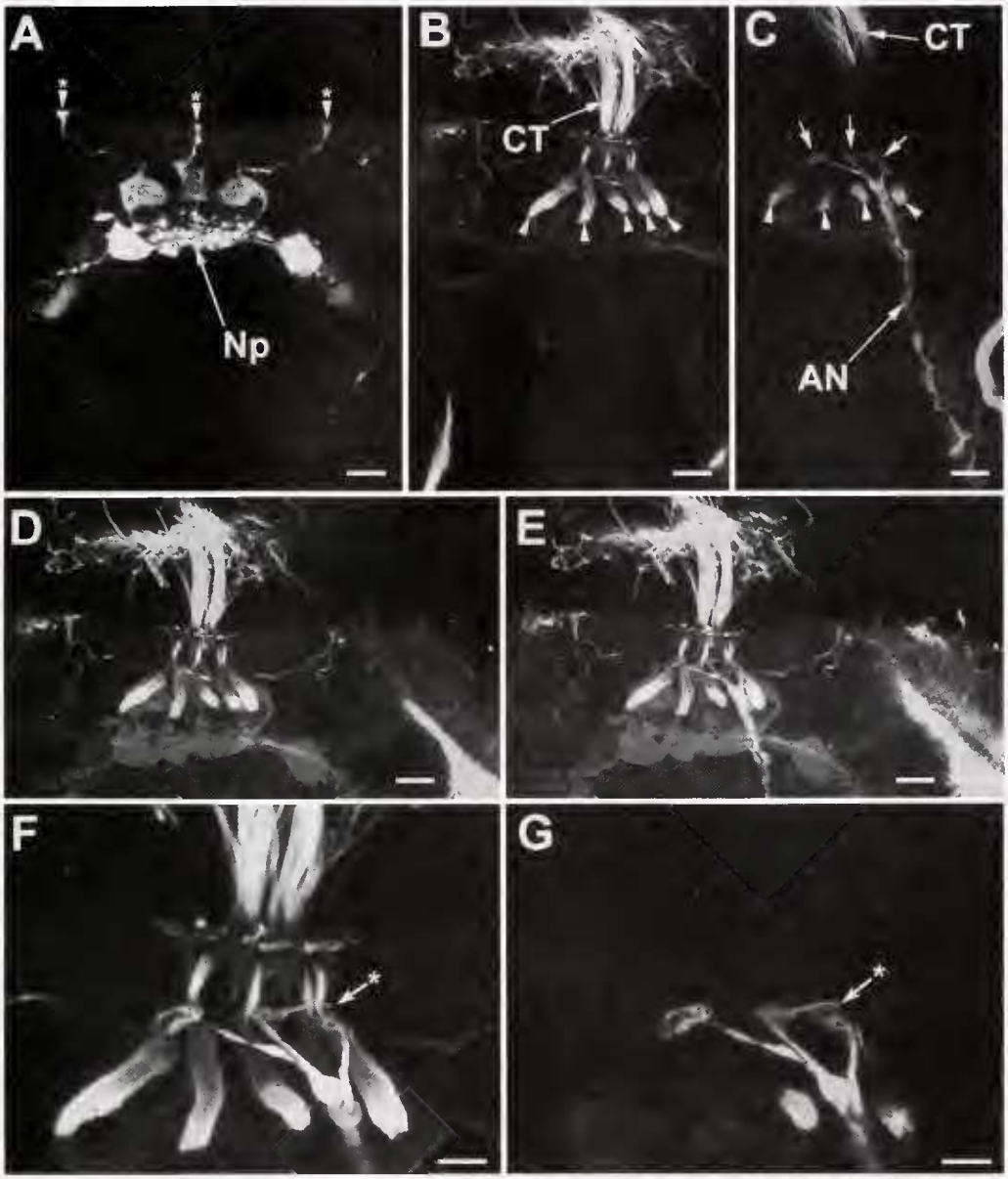

Figure 5. Known components associated with the apical ganglion and the newly described apical nerve in 13-day-old larva of *Melibe leonina*. All images are from a frontal view as seen from the dorsal side of the same larva. (A) Serotonergic neurons in the apical ganglion. The perikarya of the five serotonergic neurons are brightly labeled and situated with three above and one on either side of the apical ganglion neuropil (Np). The more weakly labeled neurons lateral to these are presumably in the edges of the cerebral ganglia. Asterisk-arrowheads identify the dendritic termini of the three sensory serotonergic neurons. (B) Ampullary neuron ciliary bundles (arrowheads) and ciliary tuft (CT) cells in the apical ganglion. (C) The apical nerve (AN) extends from the visceral region of the larva (not in picture) to a position slightly dorsal to the ampullary neuron ciliary bundles, the bases of which are identified by the arrowheads. Beneath the pretrochal epithelium, the end of the apical nerve splits into three branches with swollen termini (arrows). Note the irregular twisted appearance of the apical nerve. A portion of the ciliary tuft (CT) is visible at the top of the picture. (D) Double labeling of the ciliary bundles of the ampullary neurons (bright white) situated adjacent and slightly dorsal to the sensory serotonergic neurons (grey). (E) The terminal branches of the apical nerve are dorsal to the ampullary neuron ciliary bundles. (F) High-magnification image of the ciliary bundles of the ampullary neurons. The ampullary canals project to the pretrochal surface and are grouped around the base of the ciliary tuft. The terminal branches of the apical nerve are visible dorsal to the ampullary neurons. A narrow appendage extends to the right from the swollen terminus of the central terminal branch (asterisk-arrow). (G) A projection constructed from the optical sections that pass through the terminal branches of the apical nerve illustrates that this process is dorsal to the narrow necks of the ampullary neuron ciliary bundles. The bases of two ciliary bundles can be seen below the terminal branches and on either side of the apical nerve. Scale bars: A–E = 10 μm; F, G = 5 μm.

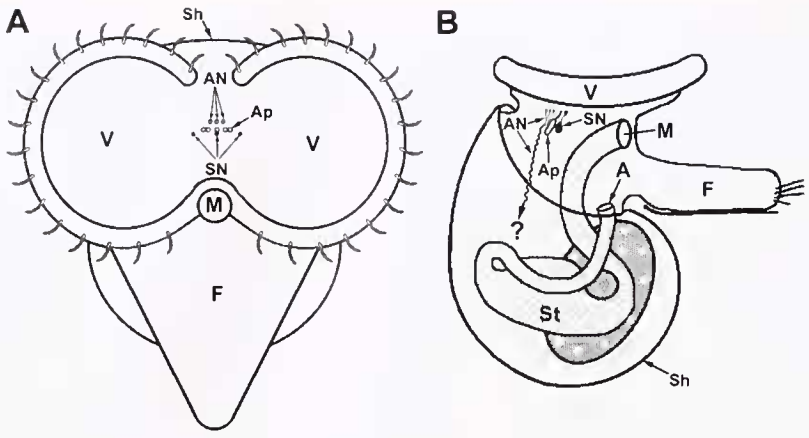

Figure 6. Relative positions of the serotonergic parampullary neurons, ampullary neurons, and apical nerve. (A) Transverse view showing the dendrites of the serotonergic neurons (SN) as most ventral, with the pores of the ampullary canals (Ap) directly above and the terminal branches of the apical nerve (AN) most dorsal. (B) Sagittal view showing the apical nerve extending toward an unknown origin in the visceral region. A, anus; F, foot; M, mouth; Sh, shell; St, stomach; V, velum..

cephalaspid and pulmonate veligers. We observed either few or no labeled cilia within the ampullary canal of newly hatched planktotrophic larvae, whereas tubulin labeling was strong in the ampullary canals of later stage planktotrophic larvae and newly hatched lecithotrophic larvae. Nonetheless, we never observed labeled cilia projecting through the ampullary pore and above the pretrochal epithelium. Assuming that the chemosensory function hypothesized for these cells is correct (Bonar, 1978; Chia and Koss, 1984; Hadfield *et al.*, 2000), the presence of this pore is essential if receptors for chemical ligands reside on the membranes of cilia within the ampulla. The very small size of the pore would augment the ability to discriminate between suitable and unsuitable habitats by effectively increasing the concentration of inducer necessary to elicit a response from the ampullary receptor neurons. An analogous situation is seen in mammalian tastebuds, where molecules defining specific taste sensations must enter a small taste pore to contact receptors on taste cell microvilli (Junqueira and Carneiro, 2003).

Larval organs and tissues present at hatching in both planktotrophic and lecithotrophic larvae are oriented toward sustaining the larva through a planktonic period that may include both feeding and growth to metamorphic competence. A number of structures critical to the metamorphic event or subsequent juvenile and adult life develop as the larva grows and attains competence; these include the radula, most ganglia of the adult nervous system, the propodium, and the primary cerata in some species (Thompson, 1958, 1962; Bonar and Hadfield, 1974; Kempf and Willows, 1977; Bickell and Kempf, 1983; Carroll and Kempf, 1994). Some such structures (*e.g.*, eyespots, digestive organs, propodium, certain nervous system components) serve multiple roles concerned with larval growth, settlement and metamorphosis, and juvenile and adult life (see Table 3). Since the cellular composition of the AG is unchanged as planktotrophic larvae of *Rostanga pulchra* grow from hatching to competence, Chia and Koss (1984) suggest that it is important both in general larval functions and in settlement and metamorphosis. It is possible that this attribute applies to specific cell types as well as to the AG as a whole. The ampullary neurons have characteristics in common with photosensory (Arendt *et al.*, 2004), mechanosensory (Emory, 1976; Altner and Prillinger, 1980), and chemosensory (Emory, 1975, 1976) receptors. They are present at hatching (and undoubtedly before), and they show little change in their structure other than small increases in the length and width of the ciliary bundles (in planktotrophic larvae) during larval life. These facts suggest that they serve one or more important sensory functions throughout the larval period. It may be that all four to five ampullary neurons mediate, for example, chemoreceptive functions related to needs during general larval life, as well as those related to sensing the metamorphic inducer once competence is attained. It is also possible that one or more of the ampullary neurons serve general sensory needs while others function in more specific modalities such as sensing the metamorphic cue. Final, though unlikely, possibilities are that some ampullary neurons act as photoreceptors or mechanoreceptors while others perform chemoreception, or that sensory modality shifts—for example, from photo- or mechanoreception to chemoreception—as the larva attains metamorphic competence.

The absence of labeling in the ampullary cilia of bivalve (*Crassostrea virginica*) and prosobranch (*Ilyanassa obsoleta, Trichotropis cancellata, Crepipatella dorsalis,* and *Fusitriton oregonensis*) larvae suggests either that the spe-

Table 2

Developmental characteristics of labeled larvae when cultured at temperatures similar to those found in their normal environment

Species (type of larval development)	Embryonic period (days)	Larval shell length: At hatching (μm)	Larval shell length: When labeled (days after hatching, μm)	Development period from oviposition to earliest competence (days)	% of development from ovi-position to competence at time of labeling*	Size at competence (μm)
Melibe leonina (planktotrophic)	10[a]	150[a]	0 d-150[a] 13 d-205[a]	42[b]	10 d-24% 23 d-55%	255[a]
Tritonia diomedea (planktotrophic)	11 (10–12[c])	145[c]	0 d-145[c] 15 d-290[c]	45[c]	11 d-24% 26 d-58%	329[c]
Janolus fuscus (planktotrophic)	?	?	?	?	?	?
Armina californica (planktotrophic)	17–23[d]	160[d]	0 d-160[d]	?	?	?
Berghia verrucicornis (lecithotrophic)	12[e]	255[e]	~0 d[‡]-255[e]	13[e]	~12d-92%	255[e]
Phestilla sibogae (lecithotrophic)	7 (6–8[f])	264[g]	~0 d[‡]-264[g]	8 (7–9[f])	~7d-~88%	264[g]
Phyllaplysia taylori (direct development‡)	27[h]	NA	?	?	?	300[h],[†]

Values were taken from the literature as indicated by the following footnotes: [a]Bickell and Kempf (1983); [b]personal observation (SCK); [c]Kempf and Willows (1977); [d]Strathmann (1987); [e]Carroll and Kempf (1990) (shell does not grow after hatching in this lecithotrophic larva); [f]Hadfield *et al.* (2000); [g]Harris (1975)—the shell does not grow after hatching in this lecithotrophic larva; [h]Bridges (1975). A question mark indicates that no measurements were taken and no published data are available; NA indicates that the measurement is not applicable for that species.

* % of development = days since oviposition ÷ days from oviposition to competence. Where a range of days for hatching is given, the mean was used to calculate % development.

† Intracapsular metamorphosis.

‡ Larvae were artificially hatched and then fixed at a time close to the normal hatching period.

cific tubulin antigen bound by the acetylated tubulin antibody used (Sigma) is "hidden" or that the antigenic makeup of ampullary cilia tubulin is different in these species. It may be that other tubulin antibodies that bind to different tubulin antigens will label the ciliary bundles in ampullary neurons of bivalves and prosobranchs. We also suspect that similar differences in antigenicity may be found in the larval ampullary cilia of other invertebrate phyla.

A final observation about labeling of ampullary neuron cilia with a tubulin antibody is that the apical canals and pores of these neurons remain closely grouped around the ciliary tuft cells throughout planktotrophic larval development. Thus, tubulin labeling within the apical canals may provide a useful landmark for determining functionally related changes in the relative position of other AG components as a larva develops to metamorphic competence.

The "apical nerve"

An unexpected result of labeling with tubulin antibody was the observation of what appears to be a nerve extending from the visceral region to a position beneath the pretrochal epithelium either in or near the AG. This putative nerve ends in three processes that presumably arise from one or more axons. The ends of these processes are swollen, suggesting that they are in contact with a cell or cells within or next to the AG. As is the case for the apical ganglion, the "apical nerve" is present in the larvae of newly hatched and later stage planktotrophs, as well as in metamorphically competent lecithotrophs. This implies that it is functionally important throughout larval life; however, its close association with the AG also suggests possible involvement in the metamorphic event.

Superficially, metamorphosis in gastropod larvae appears to involve a "simple" loss of the velum (and in some cases the shell and operculum) and rearrangement of the larval body to accommodate a benthic crawling existence; however, it also involves significant internal changes at the organ, tissue, and cellular levels that are necessary for the newly formed juvenile to survive in the adult environment. In part, these needs are met by functional activation of adult-specific organs such as the radula, primary cerata, and adult CNS ganglia that have developed in the larva prior to metamorphosis (Thompson, 1958, 1962; Bickell and Kempf, 1983). In addition, some previously used larval organs must undergo changes at the cellular and genetic level that enable them to perform needed functions in the juvenile and adult; such changes include a shift in digestive tract enzymes to accommodate a change from a vegan or lecithotrophic food source to a carnivorous life style (Kempf and Todd, 1989).

Past research provides ample evidence that metamorphic changes are, in many respects, mediated by the nervous system (Hadfield, 1978, 1998; Hadfield and Pennington, 1990). This is emphasized by the fact that Hadfield *et al.* (2000) conclusively demonstrate that the AG contains the primary receptors for the chemical cue that induces meta-

Table 3

Periods during which organs or structures of planktotrophic opisthobranch larvae appear to have a critical function

Organ or structure	Present at hatching	Pre-metamorphic larval growth and/or behavior	Settlement and metamorphosis	Juvenile and adult functions
Velum	yes	yes	no*	lost
Larval shell	yes	yes	no	lost**
Larval foot other than propodium	yes	yes	yes	yes
Statocyst	yes	yes	?	yes
Operculum	yes	yes	no	lost***
Digestive tract	yes	yes	no	yes
Nephrocysts	yes	yes	no	lost
Eye spots	no	yes	yes	yes
Larval heart	no	yes	no	lost
Propodium	no	no	yes	yes
Apical ganglion	yes	yes	yes	lost
Cerebral ganglia	yes	yes	?	yes
Pleural ganglia	no	?	?	yes
Visceral ganglion	no	?	?	yes
Pedal ganglia	no	?	yes	yes
Buccal ganglia	no	?	?	yes

Question marks indicate that there is currently no basis for the determination of function at that stage of development.

* It might be said that the slowing or cessation of velar ciliary beating causes the larva to settle; however, this appears to be under nervous control, possibly mediated by the apical ganglion (see Mackie *et al.*, 1969, 1976; Kempf *et al.*, 1997; Marois and Carew, 1997c).

** Larval shell acts as the initial template upon which the adult shell is laid down in some opisthobranch species (*e.g.*, Anaspidea).

*** May act as the initial template upon which the adult operculum is laid down in a few opisthobranch groups (*e.g.*, Pyramidellidae).

morphosis in larvae of the nudibranch *P. sibogae* and probably in the larvae of other opisthobranchs. The impetus for induction must be conveyed from the AG receptors to the organs, tissues, and cells that will undergo changes to bring about metamorphosis. While at this point the purpose of the "apical nerve" must remain the subject of conjecture, one cannot help but ask if it might be a sensory pathway that conveys the metamorphic stimulus to the visceral organs, resulting in a shift from larval to juvenile function.

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